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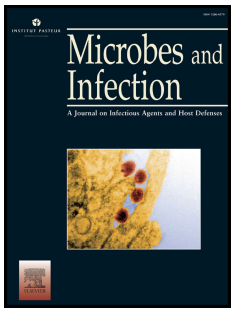

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Short communication

The *Leishmania* antigen-specific pro-inflammatory response in cutaneous leishmaniasis is linked to disease progression but not to the therapeutic failure of pentavalent antimonials

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ABSTRACT

High levels of pro-inflammatory cytokines in cutaneous leishmaniasis patients are associated with tissue damage and ulcer development. We found higher levels of TNF and IL-1β in peripheral blood mononuclear cell supernatants in response to soluble *Leishmania* antigen in individuals with a longer duration of disease. In addition, *L. braziliensis*-infected patients with a longer disease progression before treatment presented a shorter time to cure after treatment onset. No associations were found between the levels of the pro-inflammatory cytokines IL-6, TNF and IL-1-β and patients' response to pentavalent antimony treatment. Our data suggest that while the *Leishmania* antigen-specific pro-inflammatory cytokines investigated may lead to ulcer development, they do not influence therapeutic failure in cutaneous leishmaniasis patients.

Keywords: cutaneous leishmaniasis; therapeutic failure; cytokines.

1. Introduction

Localized cutaneous leishmaniasis (CL) is the most frequent form of tegumentary leishmaniasis and is characterized by the presence of one or more ulcerated cutaneous lesions with raised borders and a granulomatous background. Pentavalent antimony (Sb^V) has been established as the therapy of choice by the Brazilian government to treat tegumentary leishmaniasis; however, up to 70% of the individuals fail therapy, depending on the clinical presentation of the disease [1-3]**.**

The literature has described Th1 as the desirable response to kill *Leishmania* through the production of IFN-γ, reactive oxygen species (ROS) and nitric oxide (NO) [4, 5]. However, CL patients develop skin ulcers in spite of producing high levels of IFN-γ. In addition, the presence of pro-inflammatory cytokines TNF and IL-1β have been associated with tissue damage and lesion development [6-10]. Intermediate monocytes (CD14+CD16+) are the main cells producing TNF and IL-1β in CL [9, 10]. Also, CD8+ T and NK cells infiltrate CL lesion and produce high amounts of granzyme B and perforin, contributing to tissue damage [8, 11-13]. Yet, the production of high levels of these pro-inflammatory cytokines may also contribute to parasite killing, since low parasite counts are observed in patients with CL [14, 15]. The participation of these pro-inflammatory cytokines in the pathogenesis of CL has been well-studied in both human and mouse models. Pre-treatment with Anakinra, a drug that inhibits the production of IL-1β, was shown to prevent ulcer development in a mouse model of CL [8]. The adjuvant use of Pentoxifylline, a TNF inhibitor, was shown to decrease healing time in patients with mucosal leishmaniasis, a very inflammatory form of leishmaniasis, but produced no effect in CL patients [2, 16]. Together, these data suggest that suppressing the inflammatory response before the appearance of lesions may prevent ulcer development, whereas reducing the inflammatory response after ulcer establishment does not

lead to disease improvement; this leads us to believe that therapeutic failure in CL might be associated with other factors.

The present study evaluated the peripheral blood antigen-specific immune response in CL patients. Our main finding is that patients that presented lesion for shorter time (less than 30 days) had lower levels of pro-inflammatory cytokines and longer time to cure, when compared to patients with older lesions (more than 30 days). We also found that the assessed pro-inflammatory response is not associated with poor treatment outcome of CL.

2. Materials and methods

2.1. Study design

This study received approval from the Institutional Review Board of the School of Medicine of the Federal University of Bahia and the Brazilian National Commission for Ethics in Research, under the number: CAAE: 81315517.1.0000.5577. All patients signed terms of informed consent. Twenty-two subjects were recruited from a *L. braziliensis* transmission area in northeastern Brazil. All patients had at least one classical ulcerated lesion (supplementary table 1), no history of previous treatment, and the absence of other chronic diseases or immunodeficiency. The diagnosis for CL was confirmed by Real-time quantitative PCR and *L. braziliensis* was the species encountered in all patients [17]. Patients were grouped according to the time of disease progression (up to 30 days and more than 30 days of lesion history before therapy onset). All patients were treated intravenously with Sb^V (20mg/Kg/day) for 20 or 30 days, and successful treatment was determined by complete reepithelialization, accompanied by the disappearance of the erythema, by 90 days after the onset of therapy. Therapeutic failure was determined by the need of more than one cycle of Sb^V. When necessary, patients were submitted to new round of Sb^V treatment in accordance with the healing process of each individual. Time to cure was determined as the number of

days to reach complete reepithelization after treatment onset. All patients achieved cure after one, two or three therapeutic series with Sb^V. In 13 (59.1%) patients, the therapeutic regimen consisted of only one series (20 days), and clinical cure was observed within three months after the initiation of treatment; therefore, these individuals were considered as good responders to treatment. In eight (36.4%) patients, the treatment regimen consisted of two series (20 or 30 days) due to partial healing, the presence of active ulcers or new lesions; accordingly, these individuals were considered as poor treatment responders. Just one (4.5%) patient presented treatment failure after the second Sb^V series, and was considered a having a poor treatment response, with cure achieved only nine months after the first round of treatment.

2.2. Cell cultures and ELISA for cytokines

Peripheral blood mononuclear cells (PBMCs) were separated from total blood (collected with heparin) through Ficoll-Hypaque gradient by centrifugation at 1450 rpm for 30 minutes at 25°C. PBMCs were washed twice at 1290 rpm for 10 minutes in 0.9% NaCl, counted, ressuspended in RPMI-1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% HEPES (Gibco) and gentamicin, and plated at a concentration of 3×10^6 cells/ml on 24-well plates (Thermo Fisher Scientific, Asheville, NC, USA). Cells were stimulated with soluble *Leishmania* antigen (SLA) (5 μg/ml) and cultured for 72 hours at 37°C under 5% CO_2. Supernatants were collected and stored at -70°C. Levels of pro-inflammatory cytokines IL-6, TNF and IL-1β were measured using a previously described sandwich ELISA technique (R&D Systems, Minneapolis, MN, USA).

2.3. Soluble *Leishmania* antigen (SLA) preparation

SLA was prepared from an isolate of *L. braziliensis* as previously described [18]. Briefly, promastigotes were re-suspended in lysing solution (Tris, HCL, EDTA and leupeptin),

immersed in liquid nitrogen, and subsequently thawed at 37°C. After the freeze-thaw procedure, parasites were sonicated and then centrifuged at 14,000 × g. The supernatant was filtered and assayed for protein concentrations, tested for endotoxins using the Limulus amebocyte lysate test (Thermo fisher scientific, NY, USA), and used at a concentration of 5 μg/ml.

2.4. Statistical analysis

Wilcoxon's matched pair signed rank test was used to compare cytokine levels among the different conditions within a given group. To determine group differences, we employed the Mann–Whitney test, while non-parametric (Spearman's) correlation analysis was used to evaluate relationships between two variables. To investigate associations between cytokine levels and poor treatment outcome, groups were divided into high and low producers based on median cytokine levels and relative risk calculations. Differences were considered significant when *p*-value was <0.05. Prism version 5 for Windows (GraphPad Software, San Diego, CA) was used for statistical analyses. Sample size was determined by comparing the frequency of individuals who had less than 30 days of disease and cured after one cycle of Sb^V with the frequency of those with more than 30 days of disease who cured after a single cycle of Sb^V. An α □□□□r of 0.05 and a power of 90% were used to determine sample size.

3. Results

The demographic and clinical parameters of all included individuals, as well as their responses to treatment, are detailed in Table 1. Age, gender, number of lesions and LST size were all found not to be associated with treatment outcome (Table 1). The literature shows that individuals in the very early phase of disease, i.e. before the appearance of ulcers, are more likely to fail Sb^V therapy [3, 19]. Here we recruited CL patients who demonstrated

ulcerated lesions and found an association between a shorter time of disease progression (up to 30 days of lesion history before therapy onset) and therapeutic failure (RR=3.5) (Table 1). Our data also show that longer healing times were associated with shorter periods of disease progression (Figure 1).

A hallmark of CL is high levels of pro-inflammatory cytokines [6, 10, 20]. To investigate whether the production of pro-inflammatory cytokines is associated with poor treatment outcome, we assessed the production of IL-6, TNF and IL-1β, cytokines possibly involved in the immunopathology in CL, prior to initiating treatment with a pentavalent antimonial. Cytokine levels in non-stimulated cultures were undetectable. As expected, the production of IL-6, TNF and IL-1β was greater in SLA-stimulated PBMC cultures in comparison to non-stimulated (data not shown), and high variability in the levels of these cytokines was observed among the patients (Figure 1). Although positive correlations were found between the levels of TNF and IL-1β and time of disease progression (Figure 1), no association between high or low pro-inflammatory cytokine levels (based on the median of each cytokine) and therapeutic failure was observed (Table 1). We also did not find correlation between the levels of SLA-induced proinflammatory cytokines with time to heal after treatment onset (data not shown).

4. Discussion

An ongoing concern regarding leishmaniasis treatment is reports of high rates of therapeutic failure in regions of *Leishmania* transmission [21, 22]. Sb^V remains the first line of treatment for tegumentary leishmaniasis in Brazil, and studies investigating the therapeutic response to this drug have reported failure rates as high as 70% depending on the stage and clinical form of disease [3, 23]. The present report studied CL patients infected with *L. braziliensis* and

found a 40.9% rate of Sb^V therapeutic failure. Although just statistically significant, probably due to the low number of patients, our data shows that CL patients with ulcerated lesions with shorter time of disease progression (before treatment onset) will take longer to cure than those with longer time of disease progression. These results corroborate data in the literature that demonstrate an association between shorter time of disease progression and poorer treatment response; however, the previously published results were obtained under different conditions with variable times of disease progression, parasite species, Sb^V dosage and routes of administration [24, 25].

The identification of markers indicative of therapeutic response is highly desirable, as alternative approaches may be employed soon after diagnosis. Possible markers to assess therapeutic failure in CL may include clinical parameters, laboratorial or immunological markers, host genetic background, parasite load and the pathogen's capacity for drug resistance. Results from the present and previous studies indicate that clinical parameters, such as lesion, lymph node and intradermal skin test size are not related to treatment outcome [19]. In contrast, several studies have documented that patients with CL who start treatment later tend to respond better to treatment [3, 23-25]. Until now, two main differences identified in early and late phases of disease are parasite burden, which is higher in lesions at earlier times of infection, and the intensity of the host immune response, which is lower at early time points. We have previously shown that intermediate monocytes are the main cells producing TNF and IL-1β in PBMCs cultures, in response to SLA and upon infection with *L. braziliensis*, and cytotoxic genes expressed in lesion are associated with therapeutic failure [10, 12, 26]. Despite a solid association between inflammatory response and disease progression, the present report found that the antigen-specific systemic host's inflammatory response did not influence therapeutic outcome [6, 8, 10, 13, 27]. Since differences in the immune response between peripheral blood and lesion site might occur, future functional

studies need to be conducted using lesion cells from CL patients. With regard to parasites, our previous report demonstrated the lack of any association between parasitic load and the area of inflammation or ulcer size, which suggested that parasite load does not play a direct role in lesion development; however, in a recent work we show that high parasites transcripts are associated with therapeutic failure [15, 26]. Accordingly, we can hypothesize that when patients initiate treatment at later stages, as the immune response is better established, lesions present a lower parasitic burden to be eliminated and, consequently, individuals are more likely to respond favorably to treatment. If this hypothesis is accurate, then parasite drug resistance factors must be at play during treatment in the early phase of disease, which is when more parasites are present, thusly making effective treatment more challenging. Drug resistance has been studied in leishmaniasis and the mechanisms proposed include parasite and host factors. On the host side, effective immune response is important, as HIV patients are more likely to fail therapy [28]. Also, individual variation in responding to drugs occurs. For instance, in visceral leishmaniasis, young age and male gender have increased relapse rates to treatment with miltefosine [29]. By studding lesion cells transcripts, we have shown that high cytotoxic genes expression, as Granzyme and Granulysin, are associated with therapeutic failure in CL patients treated with Sb^{V} [26]. On the parasite side, it has been documented *Leishmania*-induced overexpression of ATP-binding cassette (ABC) transporters, which are involved in ATP-dependent transport of a variety of molecules across biological membranes, as well as, reduction of the expression of aquaporin, proteins responsible for the internalization of Sb^{III} [30]. Therefore, we believe that studies focused on the mechanisms of drug resistance among parasite isolates should be performed.

Conflict of interest: The authors deny the existence of any conflicts of interest.

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REFERENCES

[1] Machado PR, Ampuero J, Guimarães LH, Villasboas L, Rocha AT, Schriefer A, et al. Miltefosine in the treatment of cutaneous leishmaniasis caused by Leishmania braziliensis in Brazil: a randomized and controlled trial. PLoS Negl Trop Dis 2010;4:e912.

[2] Machado PR, Lessa H, Lessa M, Guimaraes LH, Bang H, Ho JL, et al. Oral pentoxifylline combined with pentavalent antimony: a randomized trial for mucosal leishmaniasis. Clin Infect Dis 2007;44:788-93.

[3] Unger A, O'Neal S, Machado PR, Guimaraes LH, Morgan DJ, Schriefer A, et al. Association of treatment of American cutaneous leishmaniasis prior to ulcer development with high rate of failure in northeastern Brazil. Am J Trop Med Hyg 2009;80:574-9.

[4] Carneiro PP, Conceicao J, Macedo M, Magalhaes V, Carvalho EM, Bacellar O. The Role of Nitric Oxide and Reactive Oxygen Species in the Killing of Leishmania braziliensis by Monocytes from Patients with Cutaneous Leishmaniasis. PloS one 2016;11:e0148084.

[5] Novais FO, Nguyen BT, Beiting DP, Carvalho LP, Glennie ND, Passos S, et al. Human classical monocytes control the intracellular stage of Leishmania braziliensis by reactive oxygen species. J Infect Dis 2014;209:1288-96.

[6] Bacellar O, Lessa H, Schriefer A, Machado P, Ribeiro de Jesus A, Dutra WO, et al. Up-regulation of Th1-type responses in mucosal leishmaniasis patients. Infect Immun 2002;70:6734-40.

[7] Carvalho LP, Passos S, Bacellar O, Lessa M, Almeida RP, Magalhaes A, et al. Differential immune regulation of activated T cells between cutaneous and mucosal leishmaniasis as a model for pathogenesis. Parasite Immunol 2007;29:251-8.

[8] Novais FO, Carvalho AM, Clark ML, Carvalho LP, Beiting DP, Brodsky IE, et al. CD8+ T cell cytotoxicity mediates pathology in the skin by inflammasome activation and IL-1β production. PLoS Pathog 2017;13:e1006196.

[9] Passos S, Carvalho LP, Costa RS, Campos TM, Novais FO, Magalhaes A, et al. Intermediate monocytes contribute to pathologic immune response in Leishmania braziliensis infections. J Infect Dis 2015;211:274-82.

[10] Santos D, Campos TM, Saldanha M, Oliveira SC, Nascimento M, Zamboni DS, et al. IL-1beta Production by Intermediate Monocytes Is Associated with Immunopathology in Cutaneous Leishmaniasis. J Invest Dermatol 2018;138:1107-15.

[11] Campos TM, Costa R, Passos S, Carvalho LP. Cytotoxic activity in cutaneous leishmaniasis. Mem Inst Oswaldo Cruz 2017;112:733-40.

[12] Campos TM, Novais FO, Saldanha M, Costa R, Lordelo M, Celestino D, et al. Granzyme B Produced by Natural Killer Cells Enhances Inflammatory Response and Contributes to the Immunopathology of Cutaneous Leishmaniasis. J Infect Dis 2020;221:973-82.

[13] Santos Cda S, Boaventura V, Ribeiro Cardoso C, Tavares N, Lordelo MJ, Noronha A, et al. CD8(+) granzyme B(+)-mediated tissue injury vs. CD4(+)IFNgamma(+)-mediated parasite killing in human cutaneous leishmaniasis. J Invest Dermatol 2013;133:1533-40.
[14] Bittencourt AL, Barral A. Evaluation of the histopathological classifications of American cutaneous and mucocutaneous leishmaniasis. Mem Inst Oswaldo Cruz 1991;86:51-6.
[15] Saldanha MG, Queiroz A, Machado PR, de Carvalho LP, Scott P, de Carvalho Filho EM, et al. Characterization of the Histopathologic Features in Patients in the Early and Late Phases of Cutaneous Leishmaniasis. Am J Trop Med Hyg 2017.
[16] Brito G, Dourado M, Guimarães LH, Meireles E, Schriefer A, de Carvalho EM, et al. Oral Pentoxifylline Associated with Pentavalent Antimony: A Randomized Trial for Cutaneous Leishmaniasis. Am J Trop Med Hyg 2017;96:1155-9.
[17] Weirather JL, Jeronimo SM, Gautam S, Sundar S, Kang M, Kurtz MA, et al. Serial quantitative PCR assay for detection, species discrimination, and quantification of Leishmania spp. in human samples. J Clin Microbiol 2011;49:3892-904.
[18] Reed SG, Badaro R, Masur H, Carvalho EM, Lorenco R, Lisboa A, et al. Selection of a skin test antigen for American visceral leishmaniasis. Am J Trop Med Hyg 1986;35:79-85.
[19] Costa RS, Carvalho LP, Campos TM, Magalhaes AS, Passos ST, Schriefer A, et al. Early Cutaneous Leishmaniasis Patients Infected With Leishmania braziliensis Express Increased Inflammatory Responses After Antimony Therapy. J Infect Dis 2018;217:840-50.
[20] Carvalho LP, Passos S, Schriefer A, Carvalho EM. Protective and pathologic immune responses in human tegumentary leishmaniasis. Front Immunol 2012;3:301.
[21] Perez-Franco JE, Cruz-Barrera ML, Robayo ML, Lopez MC, Daza CD, Bedoya A, et al. Clinical and Parasitological Features of Patients with American Cutaneous Leishmaniasis that Did Not Respond to Treatment with Meglumine Antimoniate. PLoS Negl Trop Dis 2016;10:e0004739.
[22] Refai FW, Madarasingha NP, Fernandopulle R, Karunaweera N. Nonresponsiveness to standard treatment in cutaneous leishmaniasis: A case series from Sri Lanka. Trop Parasitol 2016;6:155-8.
[23] Machado P, Araujo C, Da Silva AT, Almeida RP, D'Oliveira Jr A, Bittencourt A, et al. Failure of early treatment of cutaneous leishmaniasis in preventing the development of an ulcer. Clin Infect Dis 2002;34:E69-73.
[24] Castro MDM, Cossio A, Velasco C, Osorio L. Risk factors for therapeutic failure to meglumine antimoniate and miltefosine in adults and children with cutaneous leishmaniasis in Colombia: A cohort study. PLoS Negl Trop Dis 2017;11:e0005515.
[25] Saheki MN, Lyra MR, Bedoya-Pacheco SJ, Antonio LF, Pimentel MIF, Salgueiro MM, et al. Low versus high dose of antimony for American cutaneous leishmaniasis: A randomized controlled blind non-inferiority trial in Rio de Janeiro, Brazil. PloS one 2017;12:e0178592.
[26] Amorim CF, Novais FO, Nguyen BT, Misic AM, Carvalho LP, Carvalho EM, et al. Variable gene expression and parasite load predict treatment outcome in cutaneous leishmaniasis. Sci Transl Med 2019;11.
[27] Campos TM, Passos ST, Novais FO, Beiting DP, Costa RS, Queiroz A, et al. Matrix metalloproteinase 9 production by monocytes is enhanced by TNF and participates in the pathology of human cutaneous Leishmaniasis. PLoS Negl Trop Dis 2014;8:e3282.
[28] Alvar J, Aparicio P, Aseffa A, Den Boer M, Cañavate C, Dedet JP, et al. The relationship between leishmaniasis and AIDS: the second 10 years. Clin Microbiol Rev 2008;21:334-59, table of contents.
[29] Ostyn B, Hasker E, Dorlo TP, Rijal S, Sundar S, Dujardin JC, et al. Failure of miltefosine treatment for visceral leishmaniasis in children and men in South-East Asia. PLoS One 2014;9:e100220.
[30] Ponte-Sucre A, Gamarro F, Dujardin JC, Barrett MP, López-Vélez R, García-Hernández R, et al. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. PLoS Negl Trop Dis 2017;11:e0006052.

311

312 **FIGURE LEGEND**

313 **Figure 1.** Correlation between the time of disease progression (in days) and time to cure (in
314 months) in CL patients (A), and between time of disease progression and levels of IL-6 (B),
315 TNF (C) and IL-1β (D). Concentration of IL-6, TNF and IL-1β was assessed by ELISA in
316 PBMCs from CL patients, cultured in the presence of SLA (5 μg/ml) for 72 hours. For
317 statistical analysis, Spearman's correlation test was used.

318

Table 1. Demographic and clinical parameters, and treatment response of CL patients. Comparison between groups of patients that received 1 and more than 1 cycle of Sb^V.

	Therapy series		p value	RR (CI 95%)
	1 cycle (n=13)	> 1 cycle (n=9)		
Age (years)	28±9	32±11	0.35	
Male	11 (85%)	8 (89%)	0.44	
Lesion number				
1	7 (54%)	8 (89%)	0.168	3.7 (0.57 to 24.35)
>1	6 (46%)	1 (11%)		
Disease duration				
≤ 30 days	2 (15%)	6 (67%)	***0.023***	***3.5 (1.18 to 10.30)***
> 30 days	11 (85%)	3 (33%)		
LST (mm)				
Weak (≤ 17mm)	8 (62%)	6 (67%)	0.805	1.14 (0.38 to 3.36)
Strong (> 17mm)	5 (38%)	3 (33%)		
IL-6				
≤ 366 pg/ml	6 (46%)	4 (50%)	0.863	0.90 (0.30 to 2.70)
> 366 pg/ml	7 (54%)	4 (50%)		
TNF				
≤ 300 pg/ml	5 (42%)	5 (55%)	0.531	0.72 (0.26 to 1.97)
> 300 pg/ml	7 (58%)	4 (45%)		
IL-1β				
≤ 65 pg/ml	4 (31%)	6 (75%)	0.083	0.30 (0.07 to 1.17)
> 65 pg/ml	9 (69%)	2 (25%)		

Age is represented by mean ± standard deviation. LST, Leishmania skin test; RR, relative risk; CI, confidence interval.